Theriogenology 81 (2014) 758–763

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Setting up equine embryo gender determination by preimplantation genetic diagnosis in a commercial embryo transfer program

C. Herrera [a,*], M.I. Morikawa [a], M.B. Bello [a], M. von Meyeren [a], J. Eusebio Centeno [a], P. Dufourq [a], M.M. Martinez [b], J. Llorente [a]

[a] *Cría Tanoira Embriones, Pilar, Buenos Aires, Argentina*
[b] *Laboratorio de Genética Aplicada, SRA., Cap. Fed., Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 30 July 2013
Received in revised form 29 November 2013
Accepted 7 December 2013

Keywords:
Preimplantation genetic diagnosis
Sex determination
embryo
Polymerase chain reaction
Horse

ABSTRACT

Preimplantation genetic diagnosis (PGD) allows identifying genetic traits in early embryos. Because in some equine breeds, like Polo Argentino, females are preferred to males for competition, PGD can be used to determine the gender of the embryo before transfer and thus allow the production of only female pregnancies. This procedure could have a great impact on commercial embryo production programs. The present study was conducted to adapt gender selection by PGD to a large-scale equine embryo transfer program. To achieve this, we studied (i) the effect on pregnancy rates of holding biopsied embryos for 7 to 10 hours in holding medium at 32 °C before transfer, (ii) the effect on pregnancy rates of using embryos of different sizes for biopsy, and (iii) the efficiency of amplification by heating biopsies before polymerase chain reaction. Equine embryos were classified by size ($\leq$300, 300–1000, and $>$1000 μm), biopsied, and transferred 1 to 2 or 7 to 10 hours after flushing. Some of the biopsy samples obtained were incubated for 10 minutes at 95 °C and the rest remained untreated. Pregnancy rates were recorded at 25 days of gestation; fetal gender was determined using ultrasonography and compared with PGD results. Holding biopsied embryos for 7 to 10 hours before transfer produced pregnancy rates similar to those for biopsied embryos transferred within 2 hours (63% and 57%, respectively). These results did not differ from pregnancy rates of nonbiopsied embryos undergoing the same holding times (50% for 7–10 hours and 63% for 1–2 hours). Pregnancy rates for biopsied and nonbiopsied embryos did not differ between size groups or between biopsied and nonbiopsied embryos within the same size group ($P > 0.05$). Incubating biopsy samples for 10 minutes at 95 °C before polymerase chain reaction significantly increased the diagnosis rate (78.5% vs. 45.5% for treated and nontreated biopsy samples respectively). Gender determination using incubated biopsy samples matched the results obtained using ultrasonography in all pregnancies assessed (11/11, 100%); untreated biopsy samples were correctly diagnosed in 36 of 41 assessed pregnancies (87.8%), although the difference between treated and untreated biopsy samples was not significant. Our results demonstrated that biopsied embryos can remain in holding medium before being transferred, until gender diagnosis by PGD is complete (7–10 hours), without affecting pregnancy rates. This simplifies the management of an embryo transfer program willing to incorporate PGD for gender selection, by transferring only embryos of the desired sex. Embryo biopsy can be performed in a clinical setting on embryos of different sizes, without affecting their

* Corresponding author. Tel.: +54 91150383949.
E-mail address: caroherr@gmail.com (C. Herrera).

0093-691X/$ – see front matter
http://dx.doi.org/10.1016/j.theriogenology.2013.12.013

viability. Additionally, we showed that pretreating biopsy samples with a short incubation at 95 °C improved the overall efficiency of embryo sex determination.

1. Introduction

Preimplantation genetic diagnosis (PGD) was first used in humans more than 20 years ago [1]. Since then, this technology has been improved and currently being successfully used by human IVF clinics to determine various genetic traits of early stage embryos [2]. In recent years, PGD has also been used in bovine and caprine embryos [3,4].

The complete genome sequence of the domestic horse was published in 2009 [5] and genes have been associated with 40 equine diseases and more than 20 phenotypes [6]. In the last few years, many authors have reported their results involving biopsy of early equine embryos [7–14]. This highlights the growing interest of incorporating this technique to the equine industry. Biopsy samples were obtained using a microblade [7,8], a Piezo drill [10,12,13], or a micropipette [14] with subsequent pregnancy rates of 21% to 40%, 50% to 83%, and 62% respectively.

In the Polo Argentino breed, females are preferred to males because of their ease of training and agility. The availability of PGD for gender determination may allow transferring only embryos from the desired sex. This can have a high economic impact on commercial programs, significantly reducing the number of recipient mares needed.

Although recent research has demonstrated that equine expanded embryos can be cryopreserved successfully [15], this technique has not yet been established as a common practice in large-scale embryo transfer programs. Therefore, gender determination would be best performed on the same day as embryo retrieval to avoid transferring embryos of the undesired sex. Choi, et al. (2010) demonstrated that equine embryos can be maintained overnight in warm holding medium before being biopsied and that this does not affect their viability, even after biopsy. These authors also showed that holding equine embryos in warm medium for 4 to 6 hours after being biopsied and before being transferred into a recipient mare does not affect pregnancy rates [10]. This becomes a useful practice for embryos that are obtained at one location, diagnosed by PGD at a different one, and sent back for transfer.

However, performance of polymerase chain reaction (PCR) may take up to 10 hours from the time of biopsy, thus it is important to know if embryos may be held for this long.

To improve the diagnosis rate of PCR, we studied the effect of incubating biopsy samples for 10 minutes at 95 °C. This is a simple and short step that induces cell lysis and thus releases DNA into the medium. By using PGD of bovine embryos, other authors have already studied the efficiency of this methodology and found that heat-induced lysis significantly improves the percentage of bovine embryos that can be diagnosed by PCR when compared with proteinase K treatment [16].

Therefore, the objective of our work was to study a number of factors affecting the efficiency of gender determination by PGD in a commercial setting, aiming to maintain this technique as simple and inexpensive as possible. The factors studied were: the time between biopsy and embryo transfer, the size of the biopsied embryo, and the effect of heating biopsy samples before PCR.

2. Materials and methods

2.1. Source of embryos

Our work was performed between September 2012 and March 2013 in Buenos Aires, Argentina. Equine embryos were obtained by artificial insemination and uterine flush using 2 L of Ringer lactate solution, 7 to 8 days after ovulation of Polo Argentino donor mares with proven fertility. Semen samples from 18 fertile Polo Argentino stallions were used for artificial insemination of mares. All embryos were measured using an eyepiece and grouped according to their diameter: $\leq$300, 300 to 1000, or $>$1000 μm. Only morphologically normal embryos (grade 1) were included in the study.

2.2. Embryo biopsy

Immediately after recovery, embryos were placed on 50-μL microdroplets of Dulbecco's-PBS supplemented with 10% fetal bovine serum and 50 μg/mL of gentamicin under mineral oil, on an inverted microscope equipped with a Nikon-Narishige micromanipulation system (Narishige). Embryos were held in place by suction of a holding pipette and the inner cell mass was placed 90° clockwise away from the holding pipette. Then, the embryo capsule was punctured with a beveled micropipette (25 μm of inner diameter; ORIGIO Humagen Pipets, Charlottesville, VA, USA), half of the blastocele fluid was suctioned to reduce the turgency of the embryo and 10 to 30 cells from the inside of the blastocele cavity and adjacent to the holding pipette were aspirated taking special care not to disturb the inner cell mass (Fig. 1). The blastocele fluid and embryonic cells were aspirated by manual suction, connecting the biopsy micropipette to a disposable syringe. Using the biopsy micropipette, each biopsy sample was placed on a different microdroplet of the same medium and transferred with no more than 1 μL of medium to a 0.2-mL DNAse-free tube containing 4 μL of DNAse-free water.

2.3. Embryo transfer

Biopsied and nonbiopsied embryos were transferred transcervically to a synchronized recipient mare either 1 to 2 or 7 to 10 hours after flushing. All embryos were maintained in Dulbecco's-PBS with 10% fetal bovine serum and 50 μg/mL of gentamicin (holding medium), in 0.5-mL straws. Each straw was placed unsealed inside a plastic tube, which was capped and protected the straws from light on a warm plate at 32 °C and laid on its side until the